Helper-Cytotoxic T Lymphocyte (CTL) Determinant Linkage Required for Priming of Anti-HIV $CD8^+$ CTL in Vivo with Peptide Vaccine Constructs

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CTL are a critical component of protective immunity against viral infections, but requirements for in vivo priming of CTL are not completely understood. Covalent linkage of a helper determinant to a CTL determinant, analogous to that required for cognate help for antibody production, does not appear to be necessary in vitro, but its necessity has not been extensively explored in vivo, especially at a molecular level. We previously defined peptides encompassing multideterminant regions of HIV-1 gp160 (cluster peptides) recognized by Th from mice and humans of multiple MHC types. To investigate the requirement for Th in the development of CTL in vivo, in the context of developing a synthetic peptide vaccine for HIV active in multiple strains of mice, we immunized with compound peptides representing an immunodominant CTL epitope, P18, of gp160, co-linearly synthesized at the C-terminus of three cluster peptides. Spleen cells from compound-peptide-immunized mice of three MHC haplotypes sharing the D^d class I MHC molecule but with different class II molecules exhibited enhanced gp160-specific $CD8^+$ CTL activity and $CD4^+$ Th. In contrast, immunization with P18 alone or a mixture of cluster peptide and P18 elicited only marginal CTL activity. These results imply a requirement for determinant linkage in CTL induction in vivo similar to that already well recognized for cognate help for antibody induction. The results also define promising peptide HIV vaccine candidates for induction of CTL, as well as neutralizing antibodies, in diverse MHC types. *Journal of Immunology,* 1994, 152: 549.

A successful antiviral peptide vaccine should be capable of eliciting Th and CTL responses as well as a neutralizing antibody response in vaccinees of multiple HLA types. MHC class I-restricted CTL appear to play a central role in the recovery from viral infection (1). Although exogenous lymphokines can substitute for T cell help in the maturation of CTL precursors in vitro, the role of Th in priming CTL in vivo still remains poorly understood, compared with Th-B cell collaboration. Although much evidence for a helper requirement in CTL induction exists (2–10), there is also evidence for CTL responses independent of help (5, 11–15). Further, no study to date has shown a necessity for help requiring covalent linkage of a helper antigenic determinant to a CTL determinant, analogous to the linkage of carrier to hapten in cognate help for B cells (reviewed in ref. 16). This lack of evidence may be due to the fact that the targets of CTL are whole cells, and immunization until recently required whole cells (or tissue grafts) or live viruses. The closest one could come to suggesting determinant linkage was to show that the helper determinant and CTL determinant had to be on the same skin graft to induce rejection (9), but this could not be explored further at the molecular level. Now that the possibility of peptide immunization for CTL induction has been demonstrated (17–21), it becomes feasible to address this question using peptides comprising both helper and CTL determinants. Although recent evidence indicated that a helper site is beneficial (10, 22, 23), it was not clear whether the helper and CTL sites needed to be linked. Indeed, uncoupled helper and CTL epitope peptides were effective in two studies (10, 23) and not tested in the other (22), but in the former studies, the mixture of helper and CTL determinant peptides was administered in incomplete Freund's

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adjuvant emulsion, which sequesters the two peptides in the same microenvironment, or was given at high dose for multiple immunizations. Here, we address the question with a single immunization using a saponin adjuvant, QS21 (24), which does not require an emulsion, allowing us to test the requirement under limiting conditions. We also address the MHC linkage of helper activity by using congenic mouse strains differing in class II MHC but sharing the H-2D^d class I molecule presenting the CTL determinant. We use these results to develop peptide vaccine candidates for eliciting anti-HIV CTL in animals of multiple MHC types.

We have previously described the construction of synthetic peptides encompassing immunodominant Th peptides spanning multideterminant regions (25) from the HIV envelope protein gp160 (26). These so-called cluster peptides, each consisting of a cluster of overlapping determinants, were found to induce in vitro T cell proliferation and cytokine production in mice and humans of multiple MHC types, respectively (26). Three cluster peptides were used in the current study: PCLUS3 (residues 421–444, KQIINMWQEVGKAMYAPPISGQIR), PCLUS4 (residues 476–499, RDNWRSELYKYKVVK IEPLGVAPT), and PCLUS6 (residues 821–853, AVAE GTDRVIEVVQGAYRAIRHIPRRIRQGLER), in which the HIV-1 IIIB numbering is according to the Los Alamos database (27), or 7 less than that of Ratner et al. (28) used in previous publications (26).

To design a peptide HIV vaccine immunogenic in multiple MHC types and to investigate the mechanisms of CTL priming in vivo, we co-linearly synthesized each of the cluster peptides at the N-terminus of an immunodominant CTL determinant, P18[2] (29) (residues 308–322, RIQRGPGRAFVTIGK), previously identified to be recognized by murine CD8$^+$ CTL of four distinct MHC types (30) as well as human T cells from HIV-infected patients representing a broad array of common HLA types (31). The P18 peptide corresponds to part of the gp160 V3 loop and principal neutralizing determinant region of gp160 (32–34), and is also presented by a class II MHC molecule (I-A^d) to Th cells in mice of appropriate MHC type (35). Our current results, using recombinant mice differing in class II MHC molecules, suggest that Th determinants must be covalently attached to the CTL determinant on a single peptide to facilitate T-T collaboration and CD4$^+$ class II-restricted help for priming of CTL response in vivo. Also, these cluster peptide-P18 constructs, recently found to elicit high titers of HIV-neutralizing antibodies (36), may be useful immune constructs to elicit CTL as well as neutralizing antibodies to HIV in individuals of multiple HLA types.

[2] Abbreviations used in this paper: P18, peptide 18 of the HIV envelope protein, residues 308–322; PCLUS, cluster peptide.

Materials and Methods

Peptide synthesis

The cluster peptide-peptide-18 constructs were synthesized on an automated peptide synthesizer (model 430A; Applied Biosystems, Foster City, CA) utilizing t-boc chemistry (37). The peptides were cleaved from the resin with HF and initially purified by size exclusion chromatography. Purification to single peaks was achieved by reverse-phase HPLC on μBondapack reverse-phase C18 columns (Waters Associates, Milford, MA).

Mice

H-2 congenic mice on the B10 background mice were purchased from Jackson Laboratories (Bar Harbor, ME) or bred in our own colony at BioCon, Inc., Rockville, MD. Mice used were 8 to 20 wk old.

Immunizations

Mice were immunized s.c. at the tail base with 20 nmol of each peptide mixed with QS21 (15 μg), the highly purified saponin fraction from the soap bark tree *Quillaja saponaria*, which retains the greatest adjuvant activity but is nontoxic (24).

CTL generation

Two weeks after a single immunization, immune spleen cells from B10.D2 (H-2^d), B10.A[5R] (H-2^{i5}), or B10.S[9R] (H-2^{t4}) mice (5 × 10^6/ml in 24-well culture plates in complete T cell medium (1:1 mixture of RPMI 1640 and EHAA medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10^{-5} M 2-ME) were restimulated for 6 days in vitro with 0.1 μM of P18 and 10% Con A supernatant-containing medium (Rat T stim; Collaborative Research, Inc., Bedford, MA).

CTL assay

Cytolytic activity of in vitro secondary CTL was measured as described previously (30) using a 6-h assay with ^{51}Cr-labeled targets, as indicated in the legends. Effectors were cocultured with peptide-pulsed targets at the indicated E:T ratios. The percent of specific ^{51}Cr release was calculated as 100 × ([experimental release − spontaneous release]/[maximum release − spontaneous release]). Maximum release was determined from supernatants of cells that were lysed by the addition of 5% Triton X-100. Spontaneous release was determined from targets cells incubated without added effector cells. The 18neo (H-2^d; class I MHC$^+$, class II MHC$^-$ BALB/c 3T3 fibroblasts transfected with the neomycin resistance gene) and 15–12 (BALB/c 3T3 fibroblasts expressing HIV-1 gp160) (29) were used as targets. The assay was performed in triplicate, with 5000 target cells/well.

Results and Discussion

Mice of three haplotypes, B10.D2 (H-2^d; A^{d}E^d), B10.A(5R) (H-2^{i5}; A^{b}E$^{b/k}$), and B10.S(9R) (H-2^{t4}; A^{s}E$^{s/d}$), differing in class II but sharing the class I D^d molecule, were immunized once s.c. at the tail base with purified saponin (QS21) containing the compound peptides PCLUS3–18, PCLUS4–18, or PCLUS6–18, or P18 alone. When their immune spleen cells were restimulated in vitro with P18 in the presence of IL-2, we obtained CTL that could kill both transfected BALB/c 3T3 fibroblast targets expressing endogenously the whole gp160 protein (called 15–12; ref. 29) (Fig. 1) but not control BALB/c 3T3 fibroblast targets (called 18neo, transfected with the neomycin resistance gene alone) (data not shown). They could also kill the gp160-negative 18neo cells pulsed with peptide P18 (Fig. 2), evidence that the killing was specific for

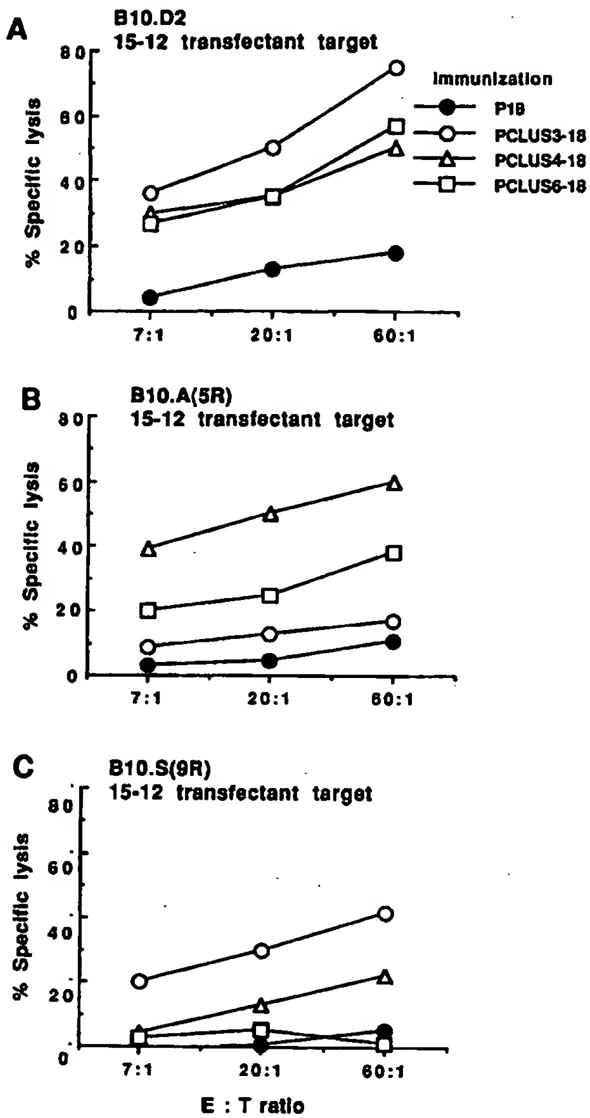

FIGURE 1. Induction of HIV-1 envelope gp160-specific CTL activity by immunization with compound peptides in QS21. Three compound peptides, PCLUS3–18, PCLUS4–18, and PCLUS6–18, were compared with free P18 for immunization of three congenic strains of mice: *A*, B10.D2; *B*, B10.A(5R); and *C*, B10.S(9R), expressing different class II MHC molecules. The fibroblast targets were BALB/c.3T3 transfectants (H-2^d, class I MHC$^+$, class II MHC$^-$) expressing the whole gp160 protein endogenously (cell line 15–12; ref. 29). Similar results were obtained with control BALB/c 3T3 cells transfected with only the neomycin resistance gene (18 neo cells) pulsed with 1 μM P18 (data not shown). As a control for both gp160 transfectants and peptide-pulsed 18 neo, background lysis using unpulsed 18 neo targets in the absence of specific peptide was less than 8% (data not shown). Effectors were cocultured with targets at the indicated E:T ratios. The assay was performed in triplicate, with 5000 target cells/well. Because SEM of triplicate wells was consistently less than 8% of the mean, error bars are omitted for clarity.

the P18 moiety. The mice primed in vivo by the compound peptides generated strong CTL activity against 15–12 as well as P18 pulsed targets at E:T ratios as low as 7:1 and reaching levels of 45 to 75% specific lysis at E:T ratios of 60:1 (Fig. 1, *A-C*). In contrast, mice immunized with P18 alone exhibited only marginal CTL activity even at maximal E:T ratios of 60:1. The shift in E:T ratio curves for killing 15–12 targets indicates more than a 10-fold greater number of CTL LU in mice primed with the compound peptide compared with mice primed in vivo with P18 alone. Non-immunized mice or mice immunized with adjuvant or peptide alone also failed to give rise to CTL (data not shown). Also, the cluster peptides without the P18 component failed to prime specific CTL (data not shown). The finding of markedly diminished or absent CD8$^+$ CTL response to P18 in mice immunized with P18 alone mixed with QS21 raised the possibility that the in vivo priming for P18 specific CTL requires CD4 class II-restricted help and that this help is provided by the immunization with the compound peptide containing cluster Th determinants.

To test whether covalent linkage of the helper determinant to the CTL determinant was required to prime mice in vivo for a CTL response, or whether a mixture of peptides not covalently linked was sufficient, we immunized B10.D2 or B10.A(5R) mice with compound peptides PCLUS3–18 or PCLUS4–18, respectively, or with mixtures of free peptides PCLUS3 or PCLUS4 and P18, or P18 alone in QS21 adjuvant (Fig. 2, *A* and *B*). The immune spleen cells were restimulated with P18 and IL-2, which compensates for any deficiency in T cell help in vitro. Cytolytic activity was measured both on the transfected 15–12 fibroblast cell line endogenously expressing gp160, and on control 18neo fibroblasts pulsed with P18 or no peptide. No lysis was observed on the control targets in the absence of peptide (data not shown). Surprisingly, the CD8$^+$ CTL activity of immune spleen cells from mice immunized with the mixture or P18 alone was negligible, whereas the compound peptide immunization elicited a strong CTL response in both strains (Fig. 2, *A* and *B*). The lysis of targets pulsed only with P18 indicates that the linkage requirement applies to induction of CTL activity specifically against the P18 determinant and therefore is not due to some other activity that might be induced by the compound peptide and measured on the gp160-expressing targets (even though no CTL activity against the PCLUS3 and PCLUS4 helper peptides was detectable; data not shown). These results indicate that covalent linkage of the cluster peptides as helper sites to the CTL site in the compound peptides PCLUS3–18 and PCLUS4–18 was required for priming of CTL in vivo. This finding was consistently reproducible in three independent experiments.

Because immunization with the compound peptide might prime CD4$^+$ CTL, we determined the phenotype of the specific CTL primed by the peptides. As shown in

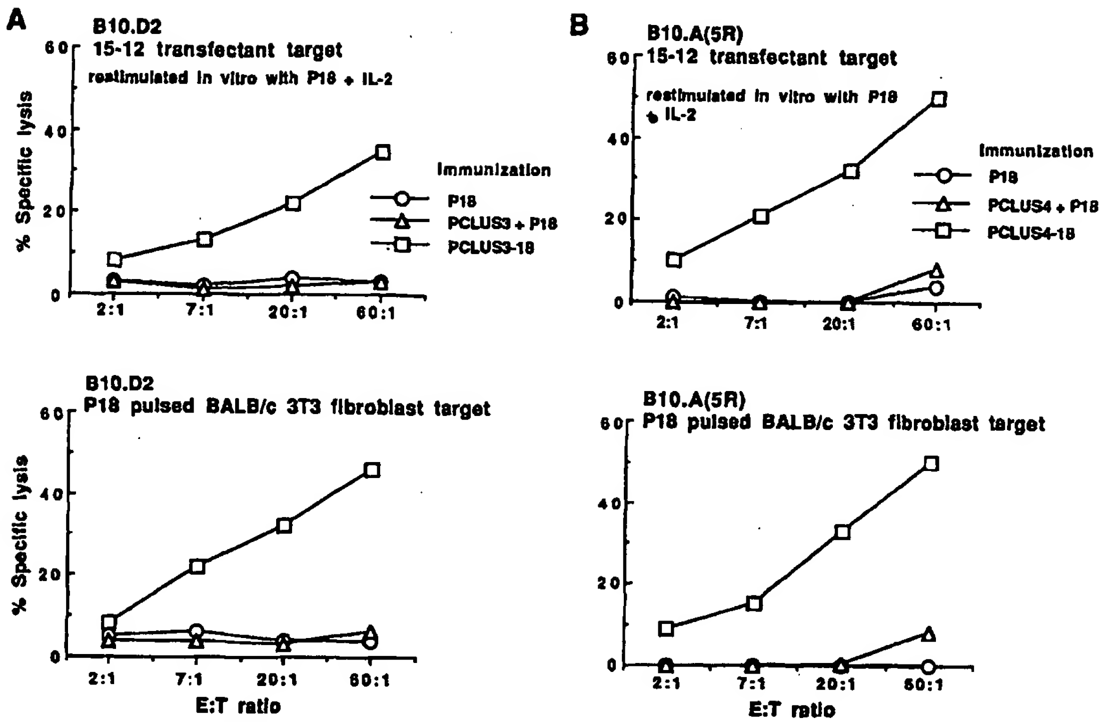

FIGURE 2. The requirement for linkage between helper and CTL determinants for priming of CTL. B10.D2 (*A*) or B10.A(5R) (*B*) mice were immunized with PCLUS3–18 or PCLUS4–18, respectively, or mixtures of PCLUS3 or PCLUS4 and P18, or P18 alone, in QS21 adjuvant. The immune spleen cells were restimulated for 6 days with 0.1 μM P18 and rIL-2 (10 U/ml, Genzyme, Cambridge, MA). The effectors were tested on the gp160 transfected line 15–12 (*upper panels*) or neo-only transfected 3T3 fibroblasts (18neo) pulsed with P18 (1 μM overnight) (*lower panels*). Background lysis on control 18neo fibroblasts in the absence of peptide was less than 8%. SEM of triplicate wells was less than 7.2% of the mean.

Figure 3*A*, the CTL were conventional $CD8^+CD4^-$ CTL. Also as the targets express only class I, not class II, MHC molecules, the CTL must be restricted by class I MHC molecules. The restriction was mapped to the D^d molecule using L cells transfected with each $H\text{-}2^d$ class I molecule (data not shown).

In contrast, the helper cells induced by the PCLUS-18 constructs were $CD4^+$, at least as measured in the in vitro stimulation of the immune spleen cells. For example, B10.A(5R) mice were immunized with 20 nmol of PCLUS4–18, and their spleen cells were treated with or without anti-CD4 plus complement before being restimulated with PCLUS4–18 or P18 alone in the presence or absence of rIL-2. The resulting effector cells were tested on the gp160-expressing 15–12 BALB/c fibroblast cell line as targets. As a control, PCLUS4 alone (not linked to P18) in the presence of IL-2 did not induce any CTL activity (data not shown). PCLUS4–18, but not P18 alone, stimulated the induction of CTL activity in the absence of rIL-2 to replace help (Fig. 3*B*). This result suggested that the PCLUS4–18 peptide was eliciting T cell help in the restimulation culture, making exogenous IL-2 unnecessary. Elimination of $CD4^+$ cells prevented the induction of CTL activity in absence of IL-2, but not in the presence of IL-2 (Fig. 3*B*). Therefore, the helper cells induced in cultures of cells immune to PCLUS4–18 and restimulated with PCLUS4–18 were $CD4^+$. The induction of CTL activity in the cultures of anti-CD4-treated cells in the presence of rIL-2 indicates that the antibody and complement treatment did not affect the CTL precursors themselves. Thus, the CTL precursors, like the CTL effector cells (Fig. 3*A*) were $CD4^-$. The induction of CTL activity in these cultures was also inhibited by the appropriate monoclonal anti-class II MHC antibodies (data not shown), confirming that the helper cells were $CD4^+$ class II MHC-restricted T cells.

In this study, we have shown a requirement for helper-CTL determinant linkage in vivo for induction of CTL, which had never been demonstrated before for CTL, in contrast to Th-B cell cooperation, for which a requirement for cognate help in vivo has been widely recognized for many years (reviewed in ref. 16). This result appears to contrast with two recent studies with mixtures of helper and CTL antigenic peptides, in which covalent linkage was not obligatory (10, 23). We may reconcile these findings by suggesting that in the former study (10), cosequestration of the peptides in an adjuvant emulsion kept them physically together in oil microdroplets, and that in the

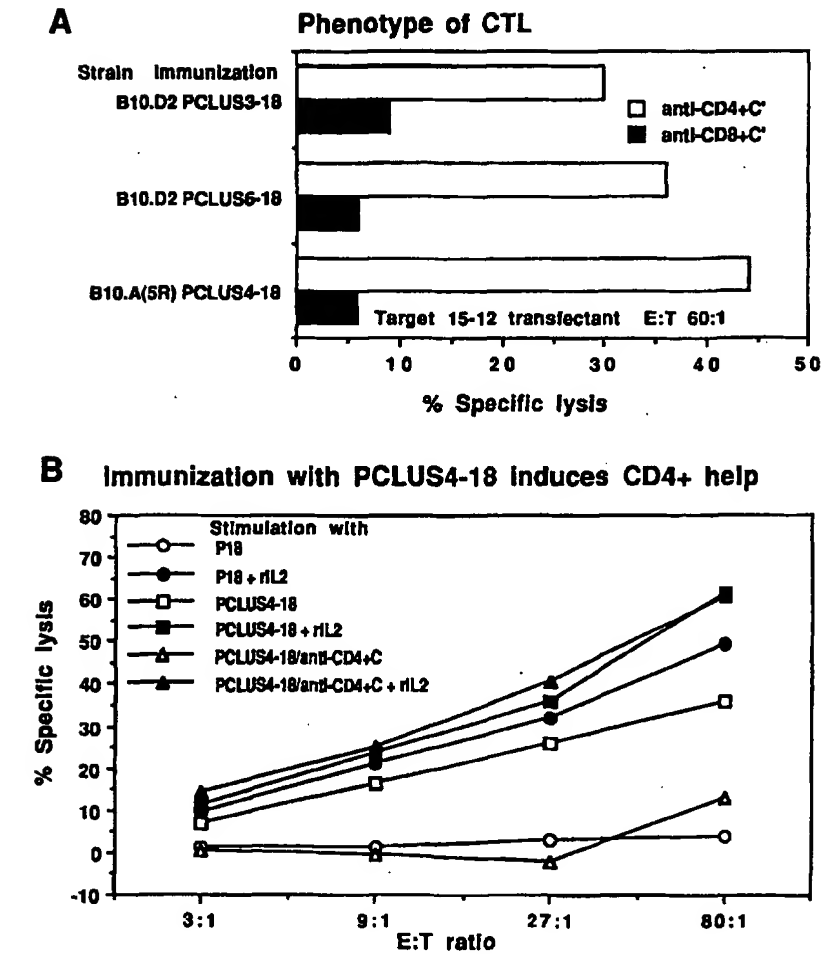

FIGURE 3. Phenotype of the CTL effectors (*A*) and the Th cells (*B*) induced by immunization with the compound peptide constructs. *A*, the CTL from B10.D2 and B10.A(5R) mice immunized with 20 nmol of the compound peptides in 15 μg QS21, after restimulation with 0.1 μM P18 plus rIL-2, were treated with either with anti-CD8 mAb (3.155; rat IgM) (48) plus complement (*solid bar*) or anti-CD4 mAb (RL.174; rat IgM) (49) plus complement (*open bar*), or complement alone (data not shown), as described previously (29), and tested on 15-12 transfectants as targets. *B*, B10.A(5R) mice immunized with 20 nmol of PCLUS4–18 in QS21, and the spleen cells were treated with anti-CD4 mAb (RL.174; rat IgM) (49) plus complement (or untreated) before stimulation for 6 days with 0.1 μM PCLUS4–18 or P18 alone in the presence or absence of 10 U/ml rIL-2. The resultant effectors were tested on the gp160-transfected cell line 15–12, or on neo-only transfected control targets (data not shown). No lysis was observed on the neo-only transfected control targets (less than 4.2%) (data not shown). SEM of triplicate wells was less than 7.3% of the mean.

latter study (23), multiple high doses of peptide used were able to overcome the inherent disadvantage of the unlinked mixture. This explanation is consistent with the requirement for proximity or presentation on the same presenting cell demonstrated by the requirement for helper and CTL determinants to be on the same skin graft to induce a rejection response in vivo (9). The explanation is also consistent with our findings in vitro (data not shown) that in the confines of a culture well, a mixture of cluster peptide and P18 is sufficient to elicit a CTL response without added IL-2 almost as efficiently as the covalent construct. The lower dose without adjuvant emulsion may more closely mimic the case in natural infection. As the peptide which induces the strongest CTL response is different from strain to strain (Fig. 1), the enhancement of CTL response by compound peptide cannot be accounted for simply by the effects of the helper site on resistance or susceptibility of the peptide to enzymatic degradation in vivo. Indeed, the reproducible difference in responsiveness to different peptide constructs among congenic recombinant mouse strains differing in class II but sharing the same H-2D^d class I molecule implies that the Th cells are class II MHC restricted and that the cluster peptides are not presented equally by all the class II molecules. Indeed, the weakest responses of the three constructs in the experiments of Figure 1 corresponded to the weakest responses of the free cluster peptides (without P18) in stimulating a T cell proliferative response in lymph node T cells of mice immunized with rHIV-1 IIIB gp160 (PCLUS 3 in the B10.A(5R) and PCLUS 6 in the B10.S(9R) giving the poorest responses) (26), consistent with the efficacy being due to the level of CD4$^+$ T cell help. Nevertheless, the use of the cluster peptides allows much broader helper recognition among mice of different MHC types than would be elicited by single helper determinants (26).

The mechanism of induction of CTL in vivo by compound peptides is likely to be that the longer peptides are taken up by specialized class II-expressing APC, implicated in CTL induction (21, 38, 39), probably at the injection site or in draining lymph nodes, before degradation of the peptides by protease in serum or extracellular fluid, so the same cell may present both the CTL and Th epitopes through class I and class II MHC, respectively. This presentation may be more efficient than others in

which the two epitopes are presented by the different APC independently. The greater efficiency of presentation by the same APC may be because it brings the Th cell and the CTL precursor together for more effective transmission of small quantities of labile lymphokines, or, as suggested by Gill and Lafferty (40), because the APC is activated by the helper cell, and then in turn is more effective at presenting Ag to the CTL. In the former case, the presentation events to both cells would have to be close in time, whereas in the latter case, they could be separated in time. In either case, the same APC would be more efficient than two separate APC, and therefore the linked determinants would be more effective than ones that could diffuse apart once injected in vivo. This is consistent with the recent observation in which specialized APC expressing class II MHC simultaneously present extracellular Ag through both class I and class II MHC pathways to CTL and Th, respectively (39), and with the skin graft experiments cited earlier (9). QS21 may be able to penetrate cell membrane and introduce Ag into the cytoplasm, from which it can enter the MHC class I presentation pathway (24). Whichever of the above mechanisms holds, the stimulation of both cells by the same APC should facilitate the delivery of help.

Progression of HIV-1 infection toward AIDS appears to correlate with a shift from Th1 to Th2 predominance in the HIV-1 specific cytokine response (41, 42). In a previous study from our laboratory, reduction in CTL response to P18 due to concurrent schistosome infection appeared to correlate with a shift from Th1, producing IL-2, to Th2 predominance (43). Th1 cells are thought to provide the $CD4^+$ class II-restricted help for CTL priming, whereas Th2 cells may secrete cytokines that inhibit CTL generation. Therefore, immunization of HIV-1 carriers for immunotherapy should be most effective if it boosts both Th1 $CD4^+$ cells and CTL, as these compound constructs are intended to do.

We recently also showed induction of P18-specific neutralizing antibody following a single immunization in mice of four different haplotypes with these compound constructs containing cluster peptides (36). Although the immunization experiments presented here had to be carried out in experimental animals, the fact that the same epitopes are also recognized by human Th cell and CTL with more than one histocompatibility complex (HLA) class II or class I molecule (26, 31), suggests that the same approach should be applicable to human immunization. Cluster 3 and 4 have sequences that are relatively conserved among North American and European isolates of HIV-1, and cluster 6 spans the boundary between conserved and variable sequences (27). Other clusters of Th cell epitopes from other proteins to which the vaccine recipient has already been immunized, such as tetanus toxoid, could also be used as carriers to induce antibodies or CTL, but the disadvantage of this approach is that natural boosting would not occur on exposure to the pathogen, because the Th cells specific for an unrelated protein would not be restimulated by the pathogen, as has been pointed out for malaria vaccines as well (44). Although the hypervariability of the V3 loop raises concerns for the design of a vaccine aimed at eliciting neutralizing antibodies and CTL specific for this region, it is encouraging that the same constructs with the P18 region from the HIV-1 MN isolate, which is representative of the most prevalent strains in Europe and North America (45, 46), were also found to prime in vivo for a CTL response and to elicit a neutralizing antibody response against the MN variant (data not shown). Also, recently, we found that CTL populations with broad specificities could be generated by restimulation of IIIB-gp160 primed murine spleen cells with MN-type peptide with an aliphatic substitution at one position (47). Further, P18 is promiscuously recognized by human CTL with several different very common class I HLA molecules, HLA-A2 (31), HLA-A3 (50), and at least one other HLA molecule (31). Thus, these constructs that elicit efficient linked help for induction of both neutralizing antibodies and CTL are worthy of consideration as vaccine candidates for prevention or immunotherapy of AIDS, and similar approaches may be applicable to other pathogens.

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